Evaluation of *C. elegans* growth and juvenile population locomotor activity as a potential screening tool for identifying chemical hazards

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Abstract

The worm Development and Activity Test (wDAT) measures *C. elegans* developmental milestone acquisition and stage-specific spontaneous locomotor activity (SLA). Previously, the wDAT identified developmental delays (DevDel) and SLA level changes in *C. elegans* with mammalian developmental neurotoxins arsenic, lead, and mercury. Cyclophosphamide (Cp), 5-Fluorouracil (5-FU), hydroxyurea (HU), and ribavirin (Rn) are teratogens that also induce growth retardation in developing mammals. In at least some studies on each of these chemicals, fetal weight reductions were seen at exposures below those that had teratogenic effects, therefore DevDel in a small alternative whole-animal model could act as a general endpoint to flag chemicals for further testing for more specific adverse developmental outcomes. As detected with the wDAT, the lowest concentrations of 5-FU, Rn, and HU that induced developmental delays in *C. elegans* were 2.0, 2.5, and 50µg/mL, respectively. Population SLA changes were detected at 2.0, 5.0, and 70µg/mL respectively for 5-FU, Rn, and HU. Cp is a prodrug that requires bioactivation by cytochrome P450s for both beneficial and toxic effects. Cp tests as a false negative in several *in vitro* assays, and it was also a false negative with the wDAT. D-mannitol also had no effect at up to 1mg/mL, making it a wDAT true negative. Further testing will determine the utility of this *C. elegans* assay within toxicity test batteries or weight of evidence approaches to identify chemicals as potential hazards to human health.

Introduction

In passing the Reducing Animal Testing Act of 2022, the U.S. Congress reinforced the FDA's Predictive Toxicology Roadmap. The roadmap encourages the development and assessment of new approach methodologies (NAMs) for toxicological testing that may be used to inform regulatory decisions. Traditional animal studies using laboratory mammals are costly and time consuming. *Caenorhabditis elegans* is a small transparent nematode emerging as a potential alternative model for developmental toxicity testing, owing to the significant conservation between *C. elegans* and mammals in many developmental pathways. *C.elegans* can be maintained in large numbers at relatively low-cost, and their quick maturation from hatchling to egg-laying adult enables experiments to be conducted rapidly, especially when compared to mammalian studies. Studies have demonstrated that *C. elegans* are effective at predicting developmental toxicity in rats and rabbits, indicating that the model has potential to serve as a toxicity screen in tiered testing and for integration as a complement to existing *in vitro* assay test batteries.

C. elegans development progresses through 4 distinct larval stages prior to reaching adulthood, with marked periods of inactivity occurring between each stage. The worm development and activity test (wDAT) measures the spontaneous locomotor activity of synchronized populations in multi-well plates, using a wMicrotracker, which tracks activity levels by the number of infrared beam interruptions that occur in half-hour increments. Peaks and valleys in the wDAT curve indicate locomotor activity levels of the population at corresponding developmental stages. Delays in peak occurrence can indicate developmental effects on the population, while alterations in peak height can signify hyper- or hypo- activity as a result of exposure to toxicants. To minimize well-to-well variability, and obtain an accurate measure of population activity, a wDAT assessment consists of range-finding for each chemical followed by 4 independently conducted experiments, where each well of a 12-well plate is used once per condition.

This study used the wDAT to assess the effects of mammalian developmental toxicants on two *C. elegans* endpoints, juvenile developmental timing and stage specific spontaneous locomotor activity. Test articles were provided as blinded chemicals and identified by numbers during the testing phase. Only after the results were collected and analyzed were the chemicals unblinded.

Results and Discussion

Cyclophosphamide (wDAT False Negative) CAS 6055-19-2, MW 279.1

Cyclophosphamide (Cp) is prescribed in the treatment of cancers and autoimmune diseases. Cp is a pro-drug that requires activation by cytochrome-P450s (CYPs) for both therapeutic and toxic effects. In mammals, maternal Cp exposure reduces fetal growth, though effects vary by species, and Cp is a false negative in many *in vitro* assays. *C. elegans* has close homologs for the human CYPs involved in Cp activation, yet no effects were detected with the wDAT at up to 1mg/mL Cp, making it a wDAT false negative (Fig. 2A).

D-Mannitol (wDAT True Negative), CAS 69-65-8, MW 182.17

D-mannitol (D-Mn) falls in the category of "generally recognized as safe" ingredients and thus is commonly used in food and pharmaceutical manufacturing. Four out of four experiments indicated no detected effects on *C. elegans* developmental timing or stage-specific locomotor activity at any tested concentration of 125-1,000µg/mL. D-Mn is a naturally occurring sugar alcohol with no identified effects on mammalian development, therefore it was accurately detected as a negative by the wDAT (Fig. 1A, 2B).

Ribavirin (wDAT True Positive), CAS 36791-04-5, MW 244.2

Ribavirin (Rn) is an antiviral medication that can be used to treat RSV, Hep C, and some hemorrhagic fevers, but is known to cause birth defects if used during pregnancy. Rn has been well-studied in multiple animal species and its developmental toxicity is well-established. A range finding experiment for Rn found a dose response in developmental delay from 5-20µg/mL Rn as well as a loss of synchronous development at 50-200µg/mL Rn. Subsequent experiments were run with exposures of 1.25- 20µg/mL Rn. Dose response developmental delays of 7% to 36% were detected at 2.5-20µg/mL Rn, and hyperactivity of 11% to 30% above controls at 5-20µg/mL, making Rn a wDAT true positive (Fig. 1B, 2C). At 2-3 days after the assay, adults exposed from L1 to ≥ 3.75µg/mL or more Rn had reduced progeny output indicating that Rn may also be a reproductive toxin in *C. elegans*.

Hydroxyurea (wDAT True Positive), CAS 127-07-1, MW 76.05

Hydroxyurea (HU) is known to inhibit DNA synthesis and is used to treat cancers of the blood, and as a treatment for sickle cell anemia. Previous studies with rats and mice have shown decreases in body weight, increased malformations and smaller litters. In the initial range-finding experiment for HU, *C. elegans* juveniles did not grow at all when exposed to 100 and 200µg/mL HU, while 50µg/mL Hu caused a 5% delay in reaching the 3rd larval stage (L3) and a 10% decrease in population motor activity. No significant effect was detected at lower exposures of 5-20µg/mL, therefore subsequent experiments were adjusted to assess an exposure range from 30-80µg/mL Hu. There was a dose-response from 6 to 18% delay at 40-80µg/mL Hu and hypoactivity of 8 to 33% at 60-80µg/mL Hu, making HU a wDAT true positive (Fig. 2D).

5-Fluorouracil (wDAT True Positive) CAS 51-21-8, FW 130.08

5-Fluoruracil (5-FU) is known to impair cellular metabolism and viability. The initial range-finding experiment revealed no growth past the second larval stage at concentrations ranging from 5-200µg/mL. Subsequent experiments assessed 5-FU at 0.5-5µg/mL. Delays in developmental timing of 5 to 27% and reductions in population activity of 15 to 47% were detected at 2-4µg/mL 5-FU. No developmental delay or changes in spontaneous locomotor activity were observed at 0.5 or 1.0mg/mL 5-FU, however it was noted that none of the exposed cohorts at even 0.5µg/mL produced viable eggs or progeny. 5-FU is therefore a true positive with the wDAT (Fig. 2E), and by microscopy observation, a potent reproductive toxin as well. Significant *C. elegans* vulval abnormalities and intestinal prolapse were noted at 5µg/mL 5-FU exposures, though the phenomenon was not quantified. After these observations, the wDAT protocol was altered to include maintenance of dosed plates for 1-3 days beyond the 3-day wMT recording to note morphological and/or reproductive changes. Previous studies have confirmed these results showing 5-FU inhibits larval development in *C. elegans* and yields lower birth rate or fetal malformation in mice and rats.

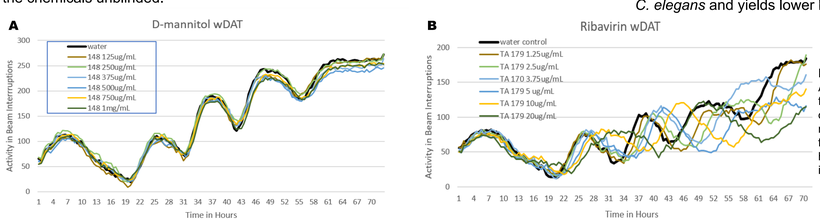

Figure 1. Example curves for individual wDAT experiments
Activity recorded as the mean of infrared beam interruptions per well (y-axis) from a single experiment over three replicate wells per condition, are graphed over half hour time increments (x-axis) **A.** All tested concentrations of D-mannitol were negative, with dosed population means showing little variance from controls. **B.** Positive results with Ribavirin show exposed populations having delays to larval peak stages and decreased activity levels that increase in a dose-response manner.

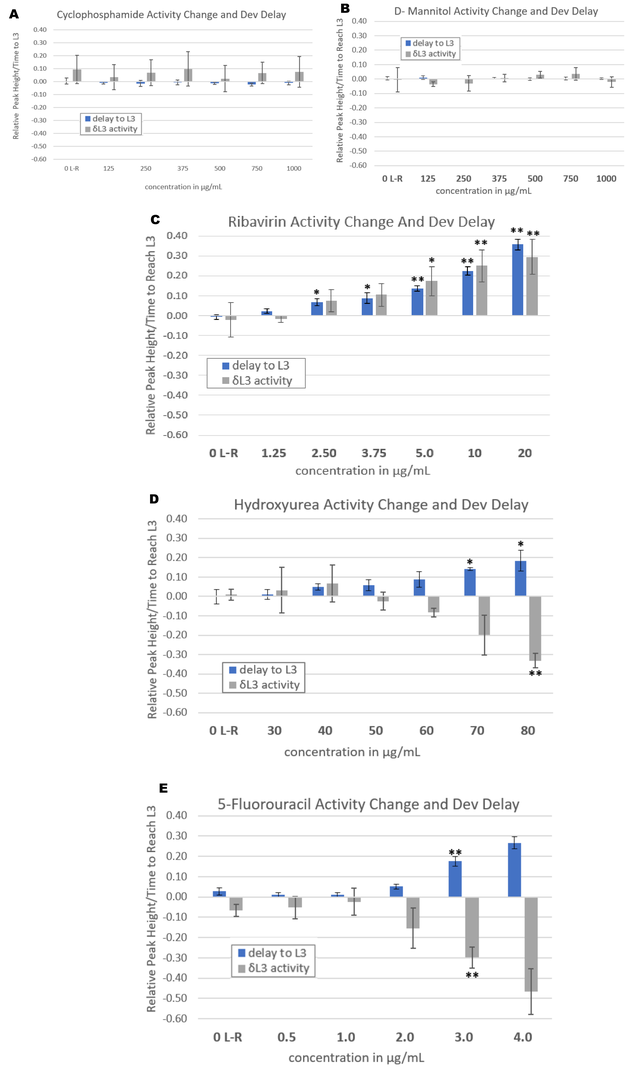

Figure 2. Analysis reveals effects on developmental timing and activity levels. Analysis of the beam interruption data generated by the wMicroTracker reveals changes in the time it takes to reach developmental stages and in the spontaneous locomotor activity levels occurring at those points. Bars and error bars represent the means and standard deviations from four independent experiments, '0 L-R' indicates the difference between simultaneous control sets of wells in separate plates run side-by-side. T-test p-values are indicated as *<0.05, ** <0.005. **A.** Cyclophosphamide, a wDAT false negative, displays no significant effects on developmental timing or activity, indicating it has not been bio-activated in *C. elegans* at tested concentrations. **B.** D-Mannitol is negative for developmental delays and changes in activity levels. **C.** Ribavirin causes increasing delays in reaching developmental timepoints as well as hyperactivity in a dose-response pattern. **D.** Hydroxyurea shows developmental delays beginning at 40µg/mL, that increases with dose, combined with hypoactivity. **E.** 5-Fluorouracil shows increases in time to reach the L3 peak combined with corresponding increasing levels of hypoactivity with increasing exposure.

The wDAT identified three out of the four chemicals in this study positive for developmental effects in mammals. Cp was a false negative for the wDAT but was correctly identified as a developmental toxicant in zebrafish embryos. In contrast, *C. elegans* identified both Hu and Rn as developmental toxicants while zebrafish tests were negative for these two chemicals. These results reinforce the need to combine new approach methodologies into batteries of tests or tiered testing strategies to better predict mammalian adverse outcomes and more reliably detect potential hazards.

Chemical Molecular Weight	CAS RN	GHS#	TTC*	wDAT Result	Zebrafish Result
5-Fluorouracil 130.08	51-21-8	positive	High (Class III)	+ve @ 15.4µM	ZDT +ve @ 1µM
Cyclophosphamide monohydrate 279.10	6055-19-2	positive	High (Class III)	-ve @ 3.58mM	ZDT +ve @ 100µM
D-mannitol 182.17	69-65-8	n.a.	Low (Class I)	-ve @ 5.49mM	n.a.
Hydroxyurea 76.05	127-07-1	positive	High (Class III)	+ve @ 657µM	ZDT -ve @ 1mM
Ribavirin 244.20	36791-04-5	positive	High (Class III)	+ve @ 5.12µM	ZDT -ve @ 1mM

+ve (orange), positive for toxicity
-ve (blue), negative for toxicity
CAS RN, Chemical Abstracts Service registration number
GHS#, Globally Harmonized System of Classification and Labelling of Chemicals, code H360 for chemicals that 'may damage fertility or the unborn child'
TTC*, predicted non-cancer threshold of toxicological concern (original Cramer rules) calculated by ChemTunes•ToxGPS
ZDT, zebrafish developmental toxicity assay at 5dpf (Song et al., 2021)
n.a., no data identified

Table 1. Comparison of NAM models for Developmental Tox Assays
In this set of four positive, one negative control for developmental toxicity, previous studies have shown that cyclophosphamide and 5-fluorouracil are true positives with zebrafish assays, but hydroxyurea and ribavirin did not have detected effects in zebrafish.. The *C. elegans* wDAT detected three of these four developmental toxins, but cyclophosphamide was a false negative. No studies on mannitol in Zebrafish were identified.

Summary Conclusions

- As measured by the wDAT, mammalian developmental toxicants 5-fluouracil, hydroxyurea, and ribavirin altered developmental timing and developmental spontaneous locomotor activity levels in juvenile *C. elegans*.
- Cyclophosphamide had no effect on developing *C. elegans* at up to 1mg/mL, making it a wDAT false negative.
- D-mannitol also had no effect at up to 1mg/mL, making it a wDAT true negative.
- This was a small study which yielded promising results for the wDAT when compared with zebrafish developmental assays. Additional studies should be conducted to determine what benefits the wDAT can offer to the future of predictive toxicology.

Mission Relevance

Chemical safety evaluations currently require data on whole-animal apical effects such as developmental toxicity. Legislation has already been passed to limit toxicity testing in vertebrate animals in some sectors, and this trend is likely to continue. No single test method can fully model toxicity effects on human development; instead, integration and translation of data derived from a variety of models and sources is the best strategy for reliable chemical safety assessment. Government agencies and non-governmental organizations are working on the best ways to improve toxicity testing and safety evaluation while shifting away from mammalian *in vivo* studies to strategies that use a combination of *in vitro* assays, *in vivo* assays with small non-mammalian organisms, and computational modeling. This study demonstrates that *C. elegans* can make a valuable contribution to weight of evidence assessments for hazard identification.

References

Song, Y.S., M.Z. Dai, C.X. Zhu, Y.F. Huang, J. Liu, C.D. Zhang, F. Xie, Y. Peng, Y. Zhang, C.Q. Li, and L.J. Zhang. 2021. Validation, Optimization, and Application of the Zebrafish Developmental Toxicity Assay for Pharmaceuticals Under the ICH S5(R3) Guideline. Front Cell Dev Biol. 9:721130. DOI:10.3389/fcell.2021.721130.